

STUDIES IN THE LIFE HISTORY AND PHYSIOLOGY
OF CERTAIN SMUTS

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INTRODUCTION

Smuts have been known for a long time as obligate parasites which develop their vegetative mycelium and chlamydospores on living hosts. Presumably, if the conditions of their parasitic life could be established under laboratory conditions, they would complete their life history equally well on artificial media. To determine what such conditions might be, selected species from material obtainable were subjected to scrutiny as to their reactions to special types of nutrition, to stimulation, temperature, and other factors. In the course of this study, morphological details in the life history of the selected smuts were also worked out as far as possible. The following species were studied: *Tilletia Tritici* (Bjerk.) Wint.; *Tilletia foetans* (B. & C.) Trel.; *Ustilago Tritici* (Pers.) Rostr.; *Ustilago Hordei* (Pers.) Kell. & Sw.; *Ustilago Zeae* (Beckm.) Unger; and *Ustilago Heufleri* Fuckel.

Since the appearance of Brefeld's (2) works, very little attention has been given to the cultivation of the smuts. Brefeld germinated the chlamydospores of a large number of species and kept them going in nutrient solutions, thus demonstrating in part their saprophytic possibilities. Here they multiplied by repeated and abundant formation of secondary spores, and the mycelium was either scanty or not formed at all. In some cases, however, e.g., *Tilletia*, in old cultures, Brefeld observed chlamydospore-like cells but did not test their capabilities. Speaking of *Ustilago Zeae*, Rawitscher (17, p. 686), in reviewing the situation, states:

Die Sporidien vermehren sich massenweise durch Sprossen, aber sie kopulieren weder (siehe darüber auch Brefeld), noch bilden sie richtige Mycelien . . . selbst in festen Nährböden.

As will be shown later in this paper, the mycelium of the corn smut can be developed and can be kept growing continuously on artificial media.

Potter (16) reports that *Sorosporium reilianum* (Kühn) McAlpine grows readily on solid nutrient media, but that "its growth is almost exclusively conidial under favorable conditions."

Kniep (12) carried through the first complete cycle from chlamydospore to chlamydospore on artificial media. He grew *Urocystis Anemones* (Pers.) Winter in a 0.1 percent malt-extract solution, and obtained chlamydospores in a few weeks' time. He considers that the saprophytically grown

chlamydospore is morphologically identical with the smut spores derived from the host. No evidence, however, appears in Kniep's paper of the ability of the spores so grown to germinate and to form the usual promycelium and sporidia. He points out, furthermore, that this malt-extract solution does not bring about the same results with some other Ustilaginales tested. From a series of concentrations of malt extract he found that a 0.5-percent solution is the optimum concentration for mycelium-formation, and it can be assumed that this smut can be grown in this way, in the vegetative condition, for any length of time.¹

INFECTION EXPERIMENTS

Tilletia Tritic (Bjerk.) Winter

The details of germination will not be taken up in this paper; numerous studies have appeared since the first account of it. Concerning infection, we have the earlier work of Kühn (13) and Wolff (21), which, although significant and thorough for the time, needs modern verification. Brefeld (2) studied the development of *Tilletia* in water and nutrient solutions. Dangeard (3), Rawitscher (18), and Paravicini (15) have given accounts of the nuclear phenomena. Most of the other work on *Tilletia* has been concerned with the control of the disease which it causes.

A logical procedure for the study of a fungous parasite is to determine how it enters the host. With this in mind, experiments were devised which would throw additional light upon the manner of infection of wheat by *Tilletia Tritic*. This smut has three kinds of spores: chlamydospores, *i.e.*, the spores that are produced on the plant; sporidia, formed at the tip of the promycelium; and secondary spores, formed by the fused sporidia. The nuclear condition of the spore is of importance, since the completion of a nuclear process may have a bearing on the time at which the chlamydospores can germinate. According to Rawitscher (18), the original fusion nucleus divides, not only twice, but three or more times within the chlamydospore, so that the promycelium has 8 or more nuclei, the sporidia receiving each one nucleus, and the fusion of pairs of these sporidia resulting in a binucleated germ tube.

In order to determine the time of infection, 1400 seeds were sterilized and potted out, a hundred to the pot. A susceptible variety of wheat, Washington Hybrid no. 128,² was used in all the infection experiments. Each successive day the seeds from one pot were removed, dusted with chlamydospores, and transplanted to the field. This was continued until the leaves of the wheat came out of the sheath, and then plats of wheat of the same age

¹ The other literature concerning the smuts has been summarized by various authors in several connections. Papers which are pertinent from the standpoint of the writer's investigation are especially those of Kniep (12), Rawitscher (17, 19), Lutman (14), Brefeld (2), Potter (16), Stakman (20), and Paravicini (15).

² Washington Hybrid 128 was sent to me by Prof. F. D. Heald of the State College of Washington, Pullman, Washington.

were dusted with spores until the plants were five or six centimeters high. The results of the experiments are given in table I.

TABLE I. *Infection of Wheat by the Chlamydospores of Tilletia Tritic*

Day Dusted	Smutted	Sound	Percent Smutted
1	93	0	100.00
2	94	0	100.00
3	90	0	100.00
4	82	11	88.16
5	70	24	74.46
6	22	64	74.42
7	10	78	11.36
8	10	81	10.98
9	2	87	2.25
10	2	89	2.20
11	0	93	.00
12	0	86	.00
13	0	91	.00
14	0	92	.00
Control	0	93	.00

The table shows that there is little or no infection when the wheat seedlings are dusted with chlamydospores ten to twelve days after planting. This is about the time it takes the leaves to grow out beyond the sheath. The greatest number of plants are infected when the seeds and the smut spores are planted together, and for the first three or four days after planting. Results indicate that infection takes place at the time of germination and diminishes rapidly after the sixth day.

A similar experiment was performed, in which only the sporidia were placed upon the wheat and wheat seedlings. The chlamydospores were germinated in pots of sterile soil or on agar media in Petri dishes. Large numbers of chlamydospores were dusted upon the surface of the medium used for germination, and the containers were placed in a saturated atmosphere. If spore balls are selected with care and broken by rolling between the sterilized thumb and index finger, allowing the spores to fall upon sterile paper, spores of such purity can be obtained that there will be very few contaminations on agar or on sterile soil. When large masses of spores germinate, the promycelia are erect and the sporidia are borne above the substratum. It is then a comparatively easy matter to place sporidia upon the wheat seeds. Moist sterile wheat seeds were picked up by means of sterile forceps and touched very lightly to the mass of sporidia; or a camel's-hair brush was used to apply the sporidia to the seedlings. The grains of wheat and the seedlings were examined under the low power of the microscope to make sure that there were only sporidia present. The results of the experiments are given in table 2.

The results can be seen to imitate closely those obtained in the first experiment.

TABLE 2. *Infection of Wheat by Sporidia of Tilletia Tritici*

Lot of 1,400 seeds planted on the same day; 100 seeds dusted with sporidia on successive days. Wheat; Washington Hybrid 128.

Day Dusted	Smutted	Sound	Percent Smutted
1	91	0	100.00
2	94	0	100.00
3	93	0	100.00
4	86	9	90.63
5	80	12	87.00
6	73	16	82.00
7	60	32	63.04
8	63	31	65.00
9	40	55	42.10
10	12	81	12.90
11	3	86	3.37
12	2	89	2.20
13	0	94	.00
14	0	90	.00
Control	0	93	.00

A third experiment was performed to determine whether or not the secondary spores and the mycelium could infect the host. A large amount of secondary spores and mycelium was obtained by making single-spore cultures from sporidia on nutrient-agar media. Many of these cultures were made in 500-cc. Erlenmeyer flasks, and, when they had developed a vigorous growth of mycelium as well as secondary spores, sterile wheat seeds were planted on the cultures. The seeds germinated and grew in the culture, and the mycelium developed around the wheat seeds and even grew up the hypocotyl to form a white mat of mycelium around the sheath (Pl. XXXIX, fig. 4). Every week some of the seedlings were transplanted to the field. The results of the experiment are given in table 3.

TABLE 3. *Effect of Saprophytic Mycelium on Wheat Seedlings*

Days in Culture	Seeds	Plants Smutted	Plants Sound
7	100	0	94
14	250	0	233
21	200	0	181
30	200	0	186
Control	100	0	88

Further infection experiments with mycelium were set up as follows:

(1) Bits of the mycelium were placed on 300 sterile wheat seeds at the time of planting. All the plants produced sound grain.

(2) The mycelium was placed on seedlings 1 to 20 days old. The plants were covered with battery jars during the first 48 to 72 hours, to insure a saturated atmosphere. All the plants produced sound grain.

The results of the experiments show that the secondary spores and the mycelium are not able to infect the host, at least at the time when the host, according to other results, is susceptible.

A great number of both free-hand and microtome sections were made of the plants that had the mycelium and secondary spores growing on them, and in no case, not even when the plant was completely covered by the mycelium, was the fungus found to enter the host. It is evident that the secondary spores and the mycelium which has been grown in culture are not able to infect the wheat plants.

The experiments with the three different kinds of spores of *T. Tritici* show very clearly that germ tubes of sporidia are instrumental in entering the host, and it is also evident that the infection takes place only at the time of germination or within a few days following.

Now the questions arise: Just how do the germ tubes from the sporidia enter the host? And at what point do they enter? To answer these questions the following method of experimentation was adopted. Wheat seeds were sterilized, dusted with chlamydospores, and planted in pots of sterile soil. Metal pots, 14 × 6 × 6 inches, one side of which could be removed, were filled with moist soil, sterilized, the side was removed, the seeds were planted on the surface of the soil, and the side was replaced. The pots were set in such a manner that the side on which the seeds were planted was in a vertical position. The advantage of this arrangement was that the conditions of growth, both for the plant and for the fungus, were favorable, and that the seedlings could be removed almost free from soil. The seedlings were mounted under the low power of the compound microscope and carefully examined.

It was found that some chlamydospores always cling to the sheath, and germinate to form a promycelium which bears sporidia at the tip. The promycelium of *Tilletia Tritici* is negatively geotropic, as it always grows upward. The sporidia are pressed tightly against the sheath and the promycelium grows up in contact with the plant. With an 8-mm. objective one can see the fused sporidia and the very fine germ tubes coming from them. The germ tube may grow along the surface of the sheath for two or three hundred microns before it enters the host. It penetrates the epidermis of the sheath, grows in between the cells, and pushes them apart, the host offering very little resistance (Pl. XL, figs. 6, 7 *a*). It is very seldom that the germ tube enters the cells of the sheath (Fig. 7 *b*), and therefore the mycelium in the sheath and in the root crown is intercellular. As soon as the tip of the germ tube enters the host, the part at the surface withers away. One must, therefore, find the tube just at the time it is entering if the connection with the sporidia is to be seen. The best time for observing this is from six to eight days after planting, if the seeds have been planted in moist soil and the temperature is about 30° C. It can also be seen very well if germinated chlamydospores are placed on seedlings 2 to 3 days old, and sections are made 50 to 80 hours afterwards.

Ustilago Zeae (Beckm.) Unger

Since the mycelium of *Tilletia Tritici* could not infect the host, it became a matter of interest to determine whether or not the mycelium of *Ustilago Zeae*, which had been obtained in culture, could infect the corn plant. *U. Zeae* was used for comparison because both Lutman (14) and Brefeld (2) have shown how the germ tube enters the host. If the sporidia of *U. Zeae* are placed on a glycerin agar, they will conjugate and form a binucleate mycelium (Pl. XXXIX, fig. 3). In a culture of this kind there are many round bodies formed that resemble chlamydospores (Pl. XL, fig. 8 c). The following experiments were performed with the mycelium:

(1) Corn was grown under sterile conditions in large 12-inch test tubes, and the mycelium was placed on plants of various ages.

(2) Plants were grown in the greenhouse, and the mycelium was placed on the growing points of the plants and on the young flowers. The young plants were placed in a saturated atmosphere for 48 hours; the growing points of the older plants were kept moist by means of absorbent cotton.

(3) In another experiment, pieces of sterile maize leaf were floated on a 5-percent solution of dextrose. Sporidia under the same conditions attached themselves to the leaves and formed a germ tube that entered the host as already described by Brefeld and Lutman.

In all these experiments the mycelium did not enter any of the plant tissues, thereby showing conclusively that the mycelium obtained in culture is unable as such to attack the host.

Ustilago Heufleri Fuckel

Ustilago Heufleri is parasitic on *Erythronium americanum*, and is one of the smuts that is very local in its distribution. It is found near Ann Arbor, in only one or two places, and it seems not to have spread beyond the very limited area where it has been known to occur for some years. The smut spores used in this study were collected in May, 1922, and the bulbs of *Erythronium* in October, 1922. The bulbs were collected in a wood one mile west of Ann Arbor, where the disease has never been known to occur. The bulbs were potted in soil, and were kept out of doors throughout the winter. When the weather became warm, the bulbs were removed to a refrigerator that was kept at a temperature of 3° C. These bulbs and smut material were used in the following experiments.

Twenty bulbs were dusted with chlamydospores at the time of planting, March 14, 1923. On May 3, sixteen of the plants of *Erythronium* had become diseased, showing pustules of chlamydospores on the leaves. The leaves of the eight plants used as controls showed no signs of infection.

In order to see whether the smut could attack the leaves of *Erythronium* when they were above ground, 160 bulbs were planted March 1, 1923, and when the leaves were about 2 cm. above the ground they were sprayed with

chlamydospores. The pots were placed in a saturated atmosphere for 48 hours, and then were placed in the greenhouse. On March 18, the greenhouse, which is usually heated to 65° F., became heated to a much higher temperature, and all the plants were killed. Up to that time, however, none of the leaves had shown any signs of infection.

A little later in the year, when the plants could be kept out of doors, the experiment was repeated. 25 bulbs were planted March 14, 1923, and were placed out of doors to grow. On May 11, when the leaves were about 2 cm. high, they were sprayed with chlamydospores, placed in a saturated atmosphere for 48 to 72 hours, and then put out of doors on the north side of the building. All the plants remained healthy and showed no signs of infection.

On May 2, 1923, twenty *Erythronium* plants were sprayed with secondary spores that had been growing in culture since November, 1922. The sporidia were transferred from the culture to a 5-percent solution of malt extract, and when they were budding rapidly they were placed on the leaves of the plants. The plants were kept in a saturated atmosphere for 48 to 72 hours, and then were placed out of doors on the north side of the building. None of the leaves became infected by the smut. This experiment shows that the secondary spores are not able to enter the leaves of the host, when the leaves are above ground.

Bits of sterile leaves of *Erythronium* were floated on 5-percent solutions of dextrose and cane sugar in which the secondary spores of *Ustilago Heufleri* were budding. The presence of the pieces of leaves caused a rapid budding of the secondary spores, and soon large numbers of them attached themselves to the surface of the leaves. Observations were made every day with an 8-mm. objective. Under the conditions described, the secondary spore forms a short germ tube at one end, and the tube enters the epidermis of the leaf before it becomes very long. Usually it is so short that it appears as though one end of the spore were pushed in between the cells. No appressorium is formed, but the cell walls are spread apart, the opening taking on a funnel-like appearance. The entrance is very similar to that of *Ustilago Zeae* as described by Brefeld (2). Observations are best made in free-hand sections of the leaves that have been infected by the secondary spores. After the germ tube has passed between the cells of the epidermis, it forms an intercellular mycelium that fills the intercellular spaces, crowding or pushing the cells apart. It is very infrequently that the germ tube enters a cell or through a stomatal opening. Large numbers of secondary spores were found attached to the cut edges of the leaf. The tubes had entered the cut or broken cells or pushed in between the cells, and soon formed an intercellular mycelium. The cultures did not last long enough to form spores, but in some cases the mycelium enlarged and broke up into segments. The segments, however, never rounded up nor became as large as mature chlamydospores, but merely became dark and formed rather heavy walls.

This experiment suggested that the germ tube might be able to infect

the leaves of *Erythronium* through wounds. The leaves of twenty plants were wounded by cutting out a small piece of the leaf or by lifting the epidermis by means of a sharp razor blade. The sporidia and secondary spores from a culture were placed on the wounds, and some of the pots of plants were placed in a saturated atmosphere for 48 to 60 hours: in other cases the wounds on the leaves were covered with paraffin. All the pots were placed out of doors. None of the leaves showed any signs of infection.

Two experiments were performed with the mycelium of *Ustilago Heufleri* that was growing in the tissue of the host. (1) A small cut was made in a healthy leaf of *Erythronium*, and a bit of the leaf that had been infected in the field was placed under the epidermis. The wounds were sealed over with paraffin, or the *Erythronium* plants were placed in a saturated atmosphere for 48 to 72 hours. (2) Portions of leaves that had been infected in the laboratory were placed under the epidermis of a healthy leaf. Both experiments gave negative results.

The results of the experiments indicate that the fungus infects the plant at the time when it is coming up through the soil, and that the leaves that are above ground are apparently able to resist the attack of the parasite. The germ tubes from the secondary spores are able to infect pieces of leaves that are floating on a sugar solution and which are probably of a greatly lowered vitality, but are apparently not able to enter a healthy growing leaf, even when the leaf has been wounded. The mycelium under the conditions of the experiment seems unable to continue its growth when transferred to a healthy leaf.

To sum up: *Tilletia Tritici* infects the young plant before the leaves break out of the sheath. The germ tube from the fused sporidia enters the sheath and forms an intercellular mycelium. Neither a germ tube from a secondary spore nor the mycelium is able to infect the host. The uninucleate sporidia and secondary spores of *Ustilago Zeae* form tubes that can infect the host at any young growing point of the plant, as shown by Brefeld (2). *Ustilago Heufleri* infects the host at the time it is growing up through the soil, and it is unable to attack leaves that are noticeably above ground.

CULTURAL LIFE HISTORY OF *TILLETIA TRITICI* AND *TILLETIA FOETANS* ON AGAR MEDIA

Brefeld (2), Stakman (20), and others who have cultivated these smuts germinated and grew them in water or in nutrient solutions. The account of Brefeld is masterly in detail and quite complete as far as it goes, and has been the classic source of information ever since his work appeared. It would seem, however, worth while to give again—this time under different cultural conditions—the story of the life history of each of these fungi as it runs its course on solid media of highly favorable nutrient constitution.

Pure cultures were obtained by means of the dilution method, or by the Kauffman spray method (10) for the isolation of a single spore. A clear

gelatin containing 2-percent malt extract or 5-percent sucrose was used in all the isolation work. The malt-extract medium is the better for general work. Single spores were transferred to the nutrient agars, and to nutrient solutions of the kind hereinafter described. The stock cultures were kept on oatmeal agar in six-inch test tubes.

These two smuts are alike in their behavior. Only the results of the experiments on *T. Tritici* will therefore be given. The chlamydospores of *T. Tritici* germinate within 2 to 3 days when placed on 2-percent malt-extract agar; under these conditions they form a non-septate promycelium, at the tip of which is borne a whorl of sporidia. There are usually eight sporidia, but the number may vary from 1 to 16 or more. The sporidia nearly always fuse in pairs before they become free from the promycelium, and may form either germ tubes or secondary spores. Two or three weeks after the germination of the chlamydospores on oatmeal agar, a mycelial layer appears. This spreads until it covers the surface of the agar by its radial growth (Pl. XXXIX, figs. 2, 4). It is white at the surface of the layer and of a fine leathery texture. It is about 0.5 to 1 cm. in thickness at the center of the mat; here also it is thickest and forms a peak. Beneath the surface, the mat is made up of a mycelium that is yellowish to light brown. After three or four months the culture takes on a brown to dark brown color, and drops of moisture collect at the surface, which has become uneven. The culture now has the characteristic odor of the stinking smut.

The mycelium rises from the fused sporidia as noted above. At first the sporidia are non-septate and are densely filled with protoplasm containing few vacuoles. They usually fuse in pairs before they are detached from the promycelium; if a single sporidium is detached it fuses with its nearest neighbor, but if too much isolated it is unable to fuse with another in the usual way. One of the pair of the fused sporidia soon sends out a slender germ tube from near its tip. At first the tube is entirely filled with protoplasm derived from the sporidia. The germ tube becomes very long, narrow, and often much coiled. It is filled with protoplasm at first, but eventually becomes empty and septate from the base outward, retaining protoplasm only at the somewhat swollen tip (Pl. XL, fig. 5 *d*). As the germ tube increases in length, the protoplasm of the sporidia first becomes vacuolate and finally disappears altogether (Pl. XL, fig. 5 *c*). At the same time 5 to 7 septa appear. The germ tube now continues to grow, with most of the protoplasm at the distal end, while cross walls appear successively on the older and lower portions, where the cells seem to have a very slight amount of protoplasm. Finally the walls of the germ tubes disintegrate.

Often, however, the fused sporidia send out short, thick hyphae instead of germ tubes (Pl. XL, fig. 5 *d*). The hyphae may have a diameter of 15 to 20 μ , and their tips are swollen and filled with protoplasm. At the base, where the hypha is attached to the sporidium, it is narrow and later becomes

septate and empty. At other times, the fused sporidia may produce crescent-shaped spores which are up to $25-45 \times 4-6 \mu$ (Pl. XL, fig. 5 c). The secondary spores are attached to the fused sporidia by very slender filaments resembling sterigmata. These secondary spores send out germ tubes or hyphae that continue to form more secondary spores. The germ tubes may also branch from the swollen tip and produce secondary spores on the branches. Often a secondary spore simply elongates into a thick filament; at first this retains the shape of the secondary spore, but later it straightens out and the protoplasm moves to its tip, while the empty region is cut off by cross walls. As the protoplasm continues to move towards the tip, the empty portion becomes multiseptate and finally disintegrates.

The large filaments formed by the sporidia and the secondary spores may branch when still quite young, but as they continue to grow the protoplasm usually passes into one of the branches, leaving the others septate and empty. The end of the filament may become enlarged and rounded, while all the protoplasmic content of the hypha passes into it. The tip is then cut off by a cross wall. Sometimes, if the hyphae are large, they break up into a series of such cells (Pl. XL, fig. 5 g), and these have very much the appearance of chlamydo-spores. Old cultures consist almost entirely of these spores, and of empty, septate filaments (Pl. XL, figs. 5 f, g).

An examination of a culture derived from a single spore in vigorous growing condition shows that it consists of two kinds of hyphae: one type is $5-8 \mu$ in thickness and $80-150 \mu$ long, and filled with coarse granular material and oil globules; the other type is very slender and long, $1-2 \times 200-400 \mu$, with very fine granular contents. In the thick hyphae the protoplasm always moves towards the tip, the lower end becoming septate and empty. As the culture becomes older and the food exhausted, the protoplasmic content may mass to form a large two-celled structure (Pl. XL, fig. 5 e). The cells of either type of mycelium may become vacuolate and finally septate and assume a bamboo-like appearance. Single-celled spore-like bodies are often formed at the tips of the branches; these occur either singly or in chains. Both the one-celled spores and the two-celled structures, mentioned above, become surrounded by heavy walls, dark brown in color. They are filled with coarse material and oil globules. These structures have, then, all the morphological characteristics of chlamydo-spores. These spores measure from 16 to 30μ in diameter. The one-celled spores germinate, when they are again supplied with nourishment, to form a branched or simple filament (Pl. XL, fig. 5 g). When two-celled bodies germinate, the wall separating the two cells is dissolved and the protoplasmic content passes into one of the two cells; this cell then forms a simple or branched hypha. The second type of mycelium is uniform in thickness, and the protoplasm usually extends throughout its length (Pl. XL, fig. 5 e); in the exceptions, series of little cells are formed, slightly larger in diameter than the filament. These structures have the appearance of oidia (Pl. XL, fig.

5 g). The fine mycelium may be considered as consisting of the younger hyphae of the fungus, because old cultures consist entirely of spore-like bodies and empty, septate, thicker hyphae.

Tilletia Grown in Solutions

Carbohydrates

In order to determine the best combination of carbohydrates for mycelial production, *Tilletia Tritici* and *T. foetans* were grown in solutions of various concentrations of the following carbohydrates: dextrose, levulose, maltose, lactose, sucrose, commercial cane sugar, and malt extract. Of course, commercial cane sugar consists mostly of sucrose, but it was considered worth while to determine any possible difference between the most highly refined and the commercial article. Malt extract is a mixture, because it is a crude extraction of malted grains; although maltose constitutes the major portion, other carbohydrates, nitrogen compounds, and salts are present in varying amounts.

T. Tritici makes its initial growth during the first 10-12 days when placed in the following sugars: dextrose, maltose, levulose, lactose, sucrose, commercial cane sugar, and malt-extract solutions of about 5-percent concentration. Under these conditions it produces a small amount of mycelium; then growth ceases, and the mycelium begins to disintegrate. Nevertheless, there is a noticeable difference of growth in the various solutions. Their order, beginning with the best, is as follows: malt extract, dextrose, commercial cane sugar, maltose, levulose, sucrose, lactose. For mycelial growth, dextrose is better than maltose and both of these are much better than sucrose and levulose. Commercial cane sugar is not quite as good as dextrose, and malt extract is far superior to any of the sugars.

The concentration for optimum growth varies for each of the carbohydrates used, as is shown in table 4.

TABLE 4. Concentration of the Carbohydrates for Optimum Growth of *Tilletia Tritici*

Carbohydrate	Concentration
Dextrose.....	M/4
Levulose.....	M/2 to M/50
Maltose.....	M/4
Sucrose.....	M/8
Lactose.....	M/8
Commercial cane sugar.....	5 percent (about M/7)
Malt extract.....	5 to 10 percent

The temperature during this experiment was kept between 20 and 25° C. Under these conditions there is very poor growth in levulose, and the minimum concentration M/50, at which there is growth, is as good as any concentration up to M/2. This sugar seem to inhibit the production of mycelium. There was a good growth of mycelium in all concentrations of malt extract from 2 to 20 percent. It was impossible to decide which

concentration is best, and 5 percent was the concentration generally used. The minimum concentration at which growth will take place in any of these sugar solutions is between M/50 and M/100, while the maximum concentration for growth is between 2 and 3 M for maltose, and 4 and 5 M for dextrose. The minimum concentration for growth in malt extract was 0.03 percent, the maximum 80 percent.

Addition of Salts to Carbohydrates

Some salts when added to sugars promote the formation of mycelium, others inhibit it. Calcium nitrate added to malt extract or dextrose produced the best mycelial growth, and potassium nitrate was next best. Potassium dihydrogen phosphate and magnesium sulfate inhibit the growth of the mycelium. At very low concentrations magnesium sulfate is beneficial to mycelium-formation. The addition of calcium nitrate and potassium nitrate to a dextrose or malt-extract solution produces good mycelial development, but if potassium dihydrogen phosphate or magnesium sulfate is added a more or less inhibiting influence is exerted, depending upon the concentration.

Full solutions with any of the sugars used as the source of carbon produce good mycelium if the concentration of potassium dihydrogen phosphate and magnesium sulfate is kept very low, M/30 to M/40.

Malt extract gives very good results with or without salts. In fact, there is little difference in growth when salts are added.

The concentration of the salts was varied, and it was found that M/20 was the best concentration for all the salts except magnesium sulfate, for which M/40 was best. All the salts inhibit the development of the mycelium when used in concentrations greater than M/10.

Horse-dung decoction, whether used alone or with sugars, was found to be a very poor medium; in most cases there was no growth at all.

Best Nutrient Solution for the Growth of the Mycelium of Tilletia

As a result of the above-described experiments, the best liquid medium for mycelial development was finally made with the following ingredients;

Calcium nitrate.....	0.4	gram
Potassium nitrate.....	0.2	gram
Potassium dihydrogen phosphate.....	0.2	gram
Magnesium sulfate.....	0.01	gram
Peptone.....	0.1	gram
Dextrose.....	2.0	grams
Malt extract.....	3.0	grams
Distilled water to 100 cc.		

As a final check on this medium, the concentration of all its constituents was also varied and tested. When the sugars are above 10 percent and salts above 5 percent, the development of the mycelium is inhibited. The most favorable concentration is about 5 percent of sugars and about 1 per-

cent of total salts, as shown above. But in no case, regardless of concentration and combination, was there any indication of the formation of chlamydospores or of spores of any other kind with these ingredients.

All cultures in the mycelial stage, when allowed to dry, turn black and the mycelium breaks up into little round cells with heavy walls. These spores will germinate to form mycelium when placed in the nutrient solution described above, or in a 3-percent solution of malt extract.

Mycelial Development of *Tilletia Tritici* on Agar Media

The development of the mycelium on agar media is better than in any of the nutrient solutions. The best agar media for the development of the mycelium are oatmeal agar, potato-sucrose agar, Leonian's agar, and malt-extract agar. Oatmeal agar is far superior to the others for mycelium-production. The largest production of mycelium takes place when the surface of the agar is moist and the atmosphere about the culture is saturated. Under such conditions the mycelium soon forms a thick white mat on the surface of the medium. As soon as the surface becomes dry, the development is checked. By "dry" is meant the condition when there is no free moisture at the surface of the agar, *i.e.*, when the water in the agar is in the adsorbed state.

The Effect of Change of Conditions on Vigorously Growing Mycelium

Temperature

Well developed cultures of *Tilletia Tritici* and *T. foetans* grown at room temperature (about 20° C.) on oatmeal agar, were placed at a temperature of 1 to 2° C. There was no change in the appearance of the fungus after three weeks. Other cultures were placed at a temperature of 25° C. At the end of a week the mycelium at the edge of the culture lost its snowy-white appearance and seemed to be dying. The center of the culture became brown and somewhat water-soaked. Examination showed that a temperature of 25° C. inhibits the development of the mycelium and causes it to disintegrate. At this temperature there is no sign of spore-formation. Some of the cultures were placed at 25° C. for 24 hours and were then placed at a lower temperature, 3 to 4° C., or left at room temperature. Changes in temperature caused no change in the kind of growth, but merely modified the rate of development.

Carbon Dioxid

The cultures of *Tilletia* were grown in an atmosphere containing various amounts of carbon dioxid. The lower percentages of carbon dioxid produced no visible change in the culture. In 75-percent carbon dioxid growth was inhibited, and in 100-percent carbon dioxid the mycelium began to disintegrate.

Oxygen

The percentage of oxygen was changed by adding pure oxygen to the tube cultures or by exhausting the tube. The pressure was decreased in the tubes by steps of 100 mm. until a pressure of 30 mm. was obtained, the lowest that could be reached with the pump at hand. There was no change in the development of the mycelium. Increase in the percentage of oxygen appears to cause no appreciable change until approximately 100 percent is reached. After a few days at this concentration of oxygen, the mycelium begins to disintegrate. Oxygen is essential to the development of the fungus, but the amount needed is supplied as readily at low as at high oxygen pressure.

CULTURAL STUDIES OF SPECIES OF USTILAGO

The genus *Ustilago* differs very greatly from the genus *Tilletia* in the physiological behavior of its species towards culture media. When spores of the species of *Ustilago* germinate, they form a promycelium that is 2- to 4-sepate and bears the sporidia at the septa. The sporidia reproduce by budding and form a large number of secondary spores. All the sporidia are uninucleate; all the secondary spores are very similar to the sporidia.

The following species of *Ustilago* were grown and studied: *U. Zeae*, *U. Hordei*, *U. Tritici*, and *U. Heufleri*.

Growth in Sugar Solutions

Solutions of dextrose, levulose, lactose, maltose, sucrose, commercial cane sugar, and malt extract were used as culture media for these smuts. The smuts were grown in solutions of maltose with concentrations varying from M/1 to M/20. A concentration of M/7 was found to be best for the budding of sporidia and for the formation of secondary spores. The results are summarized in table 5.

TABLE 5. *Development of Species of Ustilago in Various Concentrations of Maltose*
The minimum development is indicated by 1, the maximum by 4.

Maltose Concentration	<i>U. Zeae</i>	<i>U. Tritici</i>	<i>U. Hordei</i>	<i>U. Heufleri</i>
2 M.....	1	0	0	0
M/1.....	2	0	2	2
M/5.....	3	2	2	3
M/6.....	3	3	3	3
M/7.....	4	4	4	4
M/8.....	4	4	4	4
M/9.....	3	3	3	3
M/10.....	2	2	2	2
M/15.....	1	2	2	3
M/20.....	1	2	2	2

The table shows that for maltose the best concentration for the budding of the sporidia is between M/7 and M/8. One-seventh molecular has also

been found to be the optimum concentration for all the other sugars used. Sugars were sterilized at ten pounds' pressure for ten minutes.

An experiment was set up to determine the relative value of the different solutions and of a nutrient solution containing salts for the formation of secondary spores by budding. The concentration used for dextrose, levulose, maltose, and lactose was M/7; the concentration of commercial cane sugar and malt extract was 5 percent. A nutrient solution containing the following ingredients was also used:

Maltose.....	3	grams
Malt extract.....	3	grams
Potassium dihydrogen phosphate.....	1.25	grams
Magnesium sulfate.....	0.3	gram
Peptone.....	1.00	gram
Distilled water to 1,000 cc.		

The results are summarized in table 6.

TABLE 6. *Reaction of Species of Ustilago in Various Nutrient Solutions*

The minimum development is indicated by 1, the maximum by 5.

	<i>U. Zeae</i>	<i>U. Triticici</i>	<i>U. Hordei</i>	<i>U. Heufleri</i>
Dextrose.....	1	2	1	2
Lactose.....	2	2	1	1
Levulose.....	2	2	3	0
Maltose.....	5	5	5	4
Malt extract.....	5	5	5	4
Cane sugar.....	3	2	1	1
Full solution 0.2 acid*.....	5	5	5	5

* Potassium dihydrogen phosphate reacts as a buffer; the addition of small amounts of acid or alkali does not change the hydrogen-ion concentration to any appreciable extent.

The table shows that maltose is by far the best sugar for the development of the secondary spores of *Ustilago Zeae*. The secondary spores are large, and budding is very rapid. There is also some tendency to form resting spores; this takes place when the sporidia elongate, cease budding, and become swollen at one end but without the extensive formation of mycelium. There is very little budding of the secondary spores in levulose, lactose, or dextrose solutions. Dextrose is, furthermore, the lowest in nutritive value. Malt extract is better than maltose; the growth is very similar but more abundant. Cane sugar is not as good as maltose, but it is better than any of the other sugars.

Ustilago Triticici does not form sporidia in these solutions, but the promycelium breaks up into segments; therefore the comparative value of the sugar as a nutrient depends upon the number and size of the segments the fungus will form in a given solution. The table shows that maltose is much superior to the other sugars. Malt-extract solution is as good as maltose solution.

Table 6 shows that there is a slight growth of *Ustilago Hordei* in all the

sugars, but that in maltose it is much greater than in the others. In maltose the secondary spores bud rapidly and some mycelium is formed.

Ustilago Heufleri grows in all the sugar solutions except levulose, as shown by table 6. In dextrose and lactose solutions the secondary spores are very short and budding is infrequent. In maltose solution the budding is very rapid and many mycelial threads are formed. In cane sugar there is little or no budding of the secondary spores, and in a few days they turn black. Some of them conjugate, the protoplasmic content of one of the secondary spores passing into the other. There is an increase in size, and a heavy-walled spore is formed.

Maltose is by far the best sugar for the growth of the secondary spores of the smuts studied. Dextrose, levulose, and lactose are seen to have very little nutritive value. Although cane sugar is a little better than the three sugars just named, it is much inferior to maltose.

In the last row of table 6 are tabulated the reactions of the smuts to the salt-containing nutrient solution described just before the table. In this full nutrient solution many of the sporidia elongated, and the tips became swollen. The protoplasm of each sporidium passed into the swollen tip while the sporidium became vacuolate and finally empty and septate. The tip was cut off by a cross wall, and the empty part usually remained attached. In the full solution there was a difference in the behavior of the segments of *Ustilago Tritici*. They became much elongated and swollen at one end, and in some cases hyphae of considerable length were formed, with frequent fusion between the segments. Resting spores were formed in much the same manner as described for *U. Zeae*. The budding of the secondary spores of *Ustilago Heufleri* is inhibited somewhat by the full solution, and most of the secondary spores form germ tubes. A large amount of mycelium, made up of short septate hyphae, is produced. It forms a large mat which is white at first but soon turns black, and the hyphae break up into heavy-walled resting spores.

The addition of salts to maltose and to malt extract appears to influence favorably mycelium- and spore-formation and to inhibit budding. The salts were added to the sugars one at a time, and it was found that potassium nitrate and calcium nitrate in small amounts, *i.e.*, 0.01 to 0.02 percent, favored budding. Potassium dihydrogen phosphate and magnesium sulfate added to the sugars inhibited budding, and this inhibiting influence was strongest when they were added to maltose, malt extract, or levulose reactions. The addition of potassium dihydrogen phosphate and magnesium sulfate to maltose favors the formation of mycelium and causes the secondary spores of *Ustilago Heufleri* and *U. Hordei*, and the segments of *U. Tritici*, to conjugate and form resting spores. The mycelium may also break up into resting spores.

Reaction of the Smuts to Acidity and Alkalinity

Most of the smuts grow best in a slightly acid medium. In order to determine the optimum reaction for the smuts, an experiment was set up, the results of which are given in table 7. An M/7 solution of maltose was made neutral to phenolphthalein, and the reaction of the different portions of it was changed by 0.1 percent of normal HCl or KOH.

TABLE 7. *Optimum Reaction for Development of the Species of Ustilago*
Minimum growth indicated by 1, maximum by 4.

M/7 Maltose Reaction	<i>U. Zeae</i>	<i>U. Triticis</i>	<i>U. Hordei</i>	<i>U. Heufleri</i>
+0.6	0	0	0	0
+0.5	1	1	1	1
+0.4	2	2	2	2
+0.3	2	2	2	2
+0.2	3	3	3	3
+0.1	4	4	3	3
0.0	4	3	3	4
-0.1	2	2	2	4
-0.2	1	2	2	3
-0.3	0	1	0	2
-0.4	0	0	0	0

The maximum acidity at which budding still continues is about 0.5 percent, while 0.2 percent alkalinity inhibits budding of the sporidia and the secondary spores. The table shows that *U. Heufleri* grows better in an alkaline solution, but at the neutral point budding is inhibited and in the alkaline solution a large amount of mycelium is formed. The results of the experiment indicate that the optimum reaction for the budding of the secondary spores of the smuts studied is 0.1 percent acid.

Alcoholic Fermentation

In order to determine whether or not the smuts ferment sugars, they were grown in the following solutions: dextrose, levulose, lactose, maltose, sucrose, commercial cane sugar, and malt extract. The sugars were sterilized at 10 pounds' pressure for 10 minutes, and experiments were set up as follows:

- (1) Fifteen cc. of each solution was placed in a glass capsule of about 25 cc. volume, to which secondary spores of the smuts were transferred. One set was kept at room temperature and another at 29° C. At the end of 14 days the solutions were tested for ethyl alcohol.
- (2) Small test tubes were nearly filled with the sugar solutions, and free access of the air was prevented by using very tight cotton stoppers and by dipping the stoppers in paraffin. One set was kept at room temperature, the other was placed in the incubator at 29° C. This latter experiment was set up because fermentation is often inhibited when the surface of the liquid is in contact with the air, or when the supply of oxygen is large.

After 14 days the solutions were tested for ethyl alcohol. The iodoform test was used to determine the presence or absence of alcohol in both cases. The test showed that none of the solutions had been fermented, since no ethyl alcohol was found.

Solid Agar Media and Chlamydospore-formation

The smuts were grown on the following nutrient-agar and gelatin media: wheat, malted wheat, oatmeal, cornmeal, glycerin, Leonian's, malt extract, sucrose, potato-sucrose, sucrose-gelatin, and malt extract-gelatin. The smuts, both the species of *Tilletia* and of *Ustilago*, grow very well on any hard medium containing carbohydrates. The best medium for the vegetative growth of *Tilletia Tritici* and *T. foetans* is a heavy oatmeal agar. Malt-extract agar and Leonian's agar are very good also. The species of *Tilletia* form mycelium, and the sporidia of *Ustilago* species bud very profusely unless conditions of temperature, aëration, reaction, etc. of the medium are unfavorable. The growth of *Tilletia Tritici* and *T. foetans* on nutrient agars has already been dealt with; at this point the reaction of the species of *Ustilago* to solid media will be taken up.

The best medium for the production of sporidia by *Ustilago Zeae*, *U. Heufleri*, *U. Tritici*, and *U. Hordei* is one containing malt extract, peptone, calcium nitrate, and potassium nitrate. The promycelium of *U. Tritici* produces branches that break up into segments. In this respect it differs from the other species of *Ustilago*, in which sporidia are borne on the promycelium. The segments, however, bud in the same manner as other sporidia, provided the reaction of the medium is neutral or slightly acid.

The favoring temperature varies for the different species. The optimum temperature for growth of *U. Heufleri* is about 20° C., and its maximum is 36° to 37° C. *U. Zeae*, *U. Hordei*, and *U. Tritici* grow best at about 30° C. All four of these species gave very good results in culture on solid agar media.

If *Ustilago Zeae* is grown in a solution of maltose, to which potassium dihydrogen phosphate and magnesium sulfate have been added, many of the secondary spores conjugate. The same results can be obtained if the actively budding, secondary spores are placed on an alkaline glycerin agar. In about ten days a thin mat of mycelium is formed (Pl. XXXIX, fig. 3). The mycelium can be transferred to filter-paper pads (Leonian method) in capsules, in which there is a solution of maltose. The mycelium will continue to grow until a large amount is formed. When a good healthy growth of mycelium has been formed, the pad is transferred to sterile distilled water and washed several times in the course of 24 to 36 hours. The pads are then transferred to sterile capsules and placed in the incubator at 30° C. The mycelium breaks up into dark segments which have the appearance of chlamydospores. If left at room temperature, the chlamydospores are not formed.

Ustilago Heufleri grows very well on agar media containing carbohydrates, and prefers a neutral reaction. On a solid medium containing any of the sugars, or preferably maltose or malt extract, the sporidia multiply very rapidly by budding. If the cultures are allowed to grow for a long time, say two or three months, at a temperature of 28° to 30° C., there are formed large numbers of chlamydospores, the development of which can be brought about in a relatively short time if the conditions for vegetative growth are made unfavorable. When, also, the budding secondary spores are placed on an agar medium containing 1 to 2 percent glycerin with an alkaline reaction of 0.1 percent, large numbers of chlamydospores are formed. The secondary spores fuse to form a mycelium that grows within the agar, while very little if any is found at the surface of the culture; this mycelium finally breaks up into chains of chlamydospores. *These are formed in such large numbers that the agar appears black with them* (Pl. XXXIX, fig. 1). The spores are very characteristic; they are of the same size and shape as the chlamydospores formed in the plant, and they have the same structure, with an episorium and endosporium. They do not seem to germinate very readily, but those which have been observed in germination form a promycelium, which is 2- to 4-septate and bears sporidia laterally. *This smut can, therefore, complete its entire morphological life history in artificial culture.*

When the chlamydospores of *Ustilago Tritici*, obtained from its host, are germinated on the surface of a nutrient agar, a promycelium is formed; in 20 to 30 hours after germination many lateral branches can be observed on its sides. Both the promycelium and its branches are septate. The branches break up into segments that continue to reproduce by budding until a large number of segments result, so that the surface of the agar becomes covered by the segments. By transferring every four or five weeks, the smut can be grown for an indefinite period. As soon as the food supply is diminished, the segments form germ tubes which may grow to a length of 400 to 500 μ . The protoplasm remains at the tip of the tube, which is always somewhat swollen, and the rest of the filament becomes empty and septate. When the food is exhausted, large, round bodies, 6 to 9 μ in diameter, are formed at the tips of the branches and at the middle. When the culture is dry, each hypha has one or two chains of such spores, which are in every detail like the chlamydospores formed on the host plant. When the chlamydospores which were obtained in culture are placed under favorable conditions, they germinate by forming a promycelium that is septate, simple or branched, and breaks up into segments in the same manner as that derived from chlamydospores of the smut when growing on its host. *The chlamydospores of U. Tritici formed in culture must be considered true chlamydospores, since they behave similarly to those formed on its host plant.*

Ustilago Hordei is grown very easily. It germinates readily on maltose agar within 10 to 12 hours, and can be obtained in pure culture without any

trouble. It forms a promycelium which is usually 3- to 5-septate. Sporidia are produced in great numbers from the tip of the promycelium and from the septa, and the sporidia continue to multiply by budding. Soon the entire surface of the culture becomes covered by secondary spores. As the culture becomes older and the food becomes exhausted, the secondary spores form germ tubes; very often there is fusion between the secondary spores. The germ tubes may become very long, their protoplasm remains at the tip, and the rest of the filament becomes empty. When the food is exhausted, chlamydospores are formed. By growing the smut under the same conditions that were used in the culture of *Ustilago Zeae*, chlamydospores can be produced in two or three weeks. The chlamydospores germinate by forming promycelia, and sporidia at the septa. Sometimes the promycelium is short, with one or two septa or none at all (Pl. XL, fig. 11 c, d). *The chlamydospores formed in culture are similar to those obtained from the natural host.*

The smuts behave like other fungi; when conditions are favorable for vegetative growth, the sporidia and secondary spores bud continuously, but as soon as conditions are unfavorable, because of lack or withdrawal of food, spore-formation takes place.

GENERAL CONSIDERATIONS REGARDING INFECTION AND CULTURE

As indicated before, there have been very few attempts made to propagate the smuts continuously on artificial media. Brefeld (2, p. 13) cultivated the secondary spores of several smuts through successive generations for over a year; but they always continued to bud secondary spores, and formed scanty mycelium and no definite chlamydospores. However (p. 158), Brefeld, in his cultures of *Tilletia Tritici* in nutrient solution, noticed swollen bodies which resembled the young stages of chlamydospores. He never was able to show that these spores reached maturity. Stakman (20, pp. 18, 28) also obtained chlamydospore-like bodies in culture. He grew *Ustilago Tritici* and *U. Hordei* in a 5-percent solution of cane sugar, and when the food was nearly exhausted spore-like bodies were formed, which germinated when placed under favorable conditions. Kniep (12, p. 290) showed that *Urocystis Anemones* (Pers.) Wint. could be grown from chlamydospore to chlamydospore as a saprophyte on a malt-extract gelatin. The results of my own experiments show that probably all the smuts can be carried through their life history saprophytically. *Ustilago Heufleri*, *U. Hordei*, and *U. Tritici*, when grown on malt-extract agar, complete their life history in culture. The chlamydospores formed in culture are physiologically like the chlamydospores formed on the host, since, on germination, they form a normal promycelium. *Ustilago Zeae*, *Tilletia Tritici*, and *T. foetans* also form chlamydospores in culture, but these spores are physiologically unlike the chlamydospores formed on the host plant. They are like them in appearance, but the chlamydospores of *U. Zeae* do not form a

typical promycelium, while the chlamydospores of *Tilletia Tritici* and *T. foetans* do form a typical promycelium; however, the latter forms no sporidia, but secondary spores only. This difference may be due to the conditions under which the spores are formed, or the chlamydospores formed in culture may represent the types of spores produced by the old sexual form of conjugation of the sporidia. Lutman (14, p. 1222) says that "it is possible that at the present time the parasitic mycelium rarely or never starts from the conjugated conidia or promycelial cells even though they represent the old sexual form of conjugation." In any case it is quite possible that further experimentation might result in the discovery of conditions under which the life cycle of these three smuts as it occurs in nature could be exactly duplicated in culture.

The mycelium formed in culture on artificial hard media nearly always arises from the fusion of the sporidia or promycelial cells. Brefeld (2, p. 158) and Stakman (20, p. 42) have already described the formation of the germ tubes and mycelia of *Tilletia Tritici* and *T. foetans* in water and in nutrient solutions. Mycelium appeared, according to them, when the food supply was low or nearly exhausted. Kniep (12, p. 300) showed that *Ustilago Tritici* and *Urocystis Anemones* formed mycelium in a 1-percent solution of malt extract. At higher concentrations, little or no mycelium is formed. All the species of *Ustilago* studied by the writer reproduce by budding when the food supply is plentiful, but as soon as the food is nearly exhausted the mycelium begins to develop. With *U. Zeae* little or no mycelium is formed even when the solutions are very dilute, but if a transfer is made to a glycerin agar, mycelium forms immediately, since glycerin is much lower in food value than dextrose, maltose, or malt extract. Klebs (11, p. 106) has shown that for *Sporodinia grandis* Link 0.5 to 1 percent of cane sugar, maltose, or levulose is equivalent to 4 percent to 5 percent of glycerin. The transfer of the secondary spores of *U. Zeae* from a sugar solution to a glycerin agar is a change from a solution with a high and qualitatively different nutritive value to a medium with a low nutritive value of different quality. *Ustilago Tritici*, *U. Heufleri*, and *U. Hordei* will form some mycelium in a 3- to 5-percent solution of malt extract. When the culture is old, however, and the food is nearly exhausted, large amounts of mycelium are formed. *Tilletia Tritici* and *T. foetans* form mycelium in water, where the only food is that furnished by the spore. In nutrient solutions the germination of the chlamydospore is not normal, and no mycelium is formed. However, if the mycelium of *Tilletia* formed in water is transferred to a nutrient solution, as, for example, one of dextrose, large quantities of mycelium are formed. On nutrient agars, especially on oatmeal agar, *T. Tritici* and *T. foetans* form large mats of mycelium which continue to grow for several months if transfers are made occasionally. In this way the mycelium can be propagated for an indefinite period in a saprophytic condition.

Here we have a marked difference between the species of *Ustilago* and of *Tilletia*; the former produce budding sporidia when the food supply is large, and mycelium when the food supply is nearly exhausted; the latter, under the conditions of plenty of nutrition, form no budding sporidia but give rise to a well developed mycelium which bears secondary spores. There is also a difference in their growth in the host plant. The vegetative mycelium of the species of *Ustilago* is generally found at the growing points of the host, where food is concentrated and easily accessible. The fungus forms short strands of mycelium, and haustoria are wanting. The vegetative mycelium of species of *Tilletia* occurs in tissues less favorable for furnishing a concentrated food supply, *i.e.*, in the leaves and stems. The intercellular spaces are large, and the smut develops an extensive mycelium, with haustoria that penetrate the cells of the host. *Tilletia* forms mycelium more readily and more abundantly than *Ustilago*, both in the host and in culture.

When the mycelium is well nourished and nutriment is withdrawn, spore-formation begins. This is true for many fungi, and was considered a fundamental principle by Klebs (11, p. 101).

A topic related to the formation of mycelium and chlamydospores is the fusion of the sporidia. The sporidia of *Tilletia Tritici* and *T. foetans* fuse before they fall from the promycelium. In "*Ustilago carbo*" Tul., *Cintractia Montagnei* Tul., and *Ustilago Tritici* there is a fusion of the sporidia and of the segments of the promycelium; while in *Ustilago Zeae* there is no fusion of the segments of the promycelium nor of the sporidia when they are grown under similar conditions. The fusion of the sporidia may represent the old sexual form of conjugation which has ceased to play an essential rôle in the life cycle of the smuts, although it still occurs regularly when the promycelial cells or the sporidia are placed under conditions that favor it. Functionally, it may be that it has been replaced by the intracellular nuclear fusion in the chlamydospore.

From the results of recent studies of the smuts, it seems probable that there are different degrees in which the nuclear fusion occurs in the different species. In *Ustilago Tragopogonis-pratensis* Pers., according to Federly (5, pp. 1-23), the nuclei of the fused conidia travel toward each other and, as a rule, seem to fuse. In the promycelial cells of the species of *Ustilago* of the cereal grains, the nuclei move toward each other, come to lie in the same cell, and occasionally fuse. My observations show that this seems to be true for *Ustilago Heufleri*. In *Ustilago violacea* (Pers.) Fuckel, as described by Harper (7, pp. 482-487), the nucleus of each sporidium remains in its place, and all that occurs between the promycelial cells, sporidia, or secondary spores is a cytoplasmic fusion. Dangeard (3) has described a somewhat similar case in *Tilletia Tritici*, where the cytoplasmic fusion of the sporidia is never followed by a nuclear fusion. In addition to these cases in which fusions of various sorts occur, there are probably others in which no

conjugation of any kind, cytoplasmic or nuclear, takes place between the sporidia or the promycelial cells.

CONCERNING THE FURTHER LIFE HISTORY OF *USTILAGO HEUFLERI*

The mode of infection by this smut, and its reactions under various cultural conditions, have been given, for comparative purposes, in the preceding pages. Here will be taken up the germination of the spores and the nuclear history in so far as the latter could be determined.

Dormancy of the Chlamydospores

The fresh spores of *Ustilago Heufleri* which were collected in May, 1922, did not germinate, and those kept over winter in the laboratory or out of doors did little better; a very few spores were observed to germinate. Fresh spores were gathered in May and June of the succeeding year, but they also failed to germinate. The following procedure, therefore, was tried, and successful germinations were obtained.

Some of the smutted leaves were allowed to dry for three or four weeks and then were placed in a moist chamber for a few days. The moist pustules were cut out and placed in test tubes. Enough distilled water was added to cover the material, and the tubes were placed in an ice bath. When the water was frozen, the tubes were removed from the ice bath and placed in the refrigerator. After two days they were removed, and the water was drained off. The spore pustules were placed in bottles, and the bottles were attached to an oxygen generator. The oxygen was allowed to pass in until all the air was replaced by oxygen. The bottles were sealed, and the spores were left in the oxygen for from 1 to 24 hours. At intervals of two hours, spores were removed from successive bottles and placed in hanging drop cultures. The spores which had been oxygenated for 10 hours germinated in 12 to 16 hours. The spores were germinated in water, dextrose solution, and on malt-extract agar. They germinated best at temperatures from 15° to 20° C., there being no germination at 30° C. or higher.

Germination in Water

The promycelium breaks through the spore wall without rupturing it. When full grown, the promycelium is 2- to 4-septate, and measures 70-80 $\times$ 4-5 μ . The sporidia are formed at the septa, but they were not observed to form secondary spores by budding. The sporidia, while still attached, or often after detachment, send out germ tubes, protoplasmic at first, but soon becoming vacuolated or entirely empty and segmented in the basal portion. After 24 hours some of the sporidia have already germinated. Most of them, however, require a much longer time. The filament at first is narrow, but as growth progresses these infection threads assume practically the same character as those sent out by the promycelial segments. The

sporidia themselves become empty after the germ tubes are well established. Very little change, if any, was observed after 4 to 5 days.

Germination in Nutrient Solution

The germination in nutrient solution is very similar to that in water except that the promycelium is somewhat larger, and the sporidia form many secondary spores by budding. They continue to bud as long as the nutriment lasts, forming large numbers of secondary spores. Upon germination the secondary spores give rise to slender germ tubes, resembling quite closely those formed in water.

Germination on Malt-extract Agar

At 20° C. the chlamydospores germinate in 20 to 24 hours. The promycelium is 2- to 3-septate, $70-80 \times 4-5 \mu$ in size, usually simple but at times with two or three branches. The sporidia immediately reproduce by budding, forming a large colony which appears at the surface of the agar. The sporidia and secondary spores continue to bud while the nutriment lasts, and the colony continues to enlarge. At first the colony is white and moist, but as it becomes older it turns brown to dark brown. After 20 to 30 days, many of the secondary spores germinate, forming germ tubes. At first the germ tube is long and filled with fine, granular protoplasm; but soon the tip becomes swollen, the protoplasm flowing into it, while the basal portion becomes septate and empty. When the nourishment is exhausted, the tip is divided into segments by definite cross walls. The segments, which are similar in every respect to chlamydospores, round up, form a heavy wall, and become dark brown. They measure from 14 to 20 μ (Pl. XLI, figs. 13 *c*, *d*), and under favorable conditions germinate to form a promycelium. Sporidia are formed at the septa (Pl. XLI, fig. 13 *e*). Many of the secondary spores may never form germ tubes, but when the food is exhausted they surround themselves with a heavy wall and become dark brown in color, while under favorable conditions they reproduce by budding. Very often the secondary spores fuse in pairs (Pl. XLI, fig. 13 *b*).

THE CYTOLOGY OF USTILAGO HEUFLERI

The cytology of the smuts is better known than their behavior in culture. There is a fairly complete account of the nuclear phenomena of the following smuts: *Ustilago Zeae*, *U. Tritici*, *U. levis* (Kell. & Sw.) Magn., *U. nuda* (Jens.) Kell. & Sw. *U. Hordei*, *U. Tragopogonis-pratensis*, *U. violacea*, *U. Avenae*, *Cintractia Montagnei*, *Doassansia Sagittariae*, *D. Alismatis*, and *Urocystis Anemones*. Partial accounts are available for *U. longissima* (Sow.) Tul., *Doassansia deformans* Setch., *Doassansia Nymphaeae*, *Entyloma Nymphaeae* (D. D. Cunn.) Setch., and *Tilletia Tritici*. Rawitscher (18) has given a coherent account of the nuclear behavior of *Tilletia Tritici*, but he

was not able to determine the nuclear condition of the secondary spores when these occur. Dangeard (3), however, states that the secondary spores are always binucleate, but this has not been verified.

Since the chlamydospores of *Ustilago Heufleri* were obtained in culture, it became a matter of interest to determine whether or not the nuclear conditions of the spores formed in culture and of those formed in the host plant were the same. The chlamydospores from cultures were germinated on malt-extract agar, in water, and in a 5-percent solution of dextrose. The germinated spores were placed on a clean slide which had been covered with a thin film of Szombdathy's fixative. A drop of strong Flemming's solution was placed on the spores and allowed to remain for 20 to 30 minutes. The killing fluid hardens the fixative, so the spores are held fast to the slide. The preparations were washed in distilled water for 1 to 2 hours, then bleached in hydrogen peroxid, and stained in Heidenhain's haematoxylin. A few of the preparations were stained in Flemming's triple stain.

The mature chlamydospore contains a single nucleus (Pl. XLI, fig. 12 *d*). When the spore germinates it forms a 2- to 4-septate promycelium. The nucleus passes into the promycelium and two divisions occur to form four nuclei, which become separated by cross walls. Each cell of the promycelium contains one nucleus (Pl. XL, fig. 9 *a*). The sporidia are formed at the septa and at the tip of the promycelium. All the secondary spores formed by budding are uninucleate. In old cultures the secondary spores fuse in pairs to form binucleate resting spores (Pl. XLI, fig. 13 *d*), and when these are placed on malt-extract agar they germinate to form a promycelium that bears sporidia. In many cases a binucleate or multinucleate mycelium is formed. When the food in the medium is exhausted the mycelium breaks up into segments, which usually fuse in pairs to form large resting spores (Pl. XLI, fig. 13 *c*). In some cases more than two segments fuse, so that the resting spores are multinucleate. At times the nuclei in each spore are very close together, but it is very difficult to determine whether or not they fuse (Pl. XLI, fig. 13 *d*). In every case of fusion the entire contents of one cell passes into the other, and there is a cytoplasmic growth in size (Pl. XLI, fig. 13 *bb*). When the chlamydospores which were formed in culture were placed on a malt-extract agar, they germinated to form a 2- to 3-septate promycelium, and sporidia were formed at the septa and at the tip of the promycelium. Many of the spores, however, formed sporidia directly.

It is rather difficult to obtain early stages in the development of the fungus in the host. When the light spots which indicate points of infection appear on the leaves, the disease is well advanced and the mycelium found there is fully developed and breaking up into spores.

The youngest pustules that could be found were cut out and placed in strong Flemming's or medium chrom-acetic killing fluid. The bottles were attached to an exhaust pump and all the air was drawn out of the pieces of leaves, allowing the killing fluid to penetrate quickly to all parts of the

material. The material was left in the fixing fluid for 20 to 24 hours and then washed in running water for 24 hours. It was passed through eighteen grades of alcohol, being left in each of the lower concentrations for about 1/2 hour, in 70, 85, and 95 percent alcohol for 12 hours each, and in absolute alcohol for 12 hours. Then cedar oil was placed in the bottle by means of a pipette, so as to make a layer at the bottom of the absolute alcohol. When the material had sunk into the cedar oil, the alcohol was decanted and fresh cedar oil was added. After 10 to 12 hours, the material was placed in xylol and imbedded in the usual manner. The material was then sectioned, stained with Heidenhain's haematoxylin, and mounted in balsam.

The mycelium found in the plant is always intercellular. It does not spread very far from the point of infection, and is never found a centimeter beyond the pustule. In the very youngest pustules a mass of mycelium is found in the intercellular spaces just below the epidermis, and the mycelium extends in all directions from the point of infection. The mycelium usually extends to the opposite epidermis, and a little farther in the other directions. The greatest growth is lengthwise of the leaf, following the tracheae, which accounts for the formation of the elongated pustules. The largest amount of mycelium is formed at the point of infection, the hyphae growing between the cells, pushing them apart, and distorting them. Finally the host cells disintegrate, and the space becomes filled with mycelium. The hyphae at the center of the pustule break up, into segments first, and segmentation proceeds towards the periphery until all the hyphae have broken up into spores. In a mature pustule no mycelium is left, and only a few hyphae are found between the cells adjoining the pustule. The entire process, from the first appearance of the pustule to the maturing of chlamydo-spores, takes place in a week or ten days.

The mycelium found in the plant is usually multinucleate, and the nuclei are nearly always associated in loose pairs (Pl. XLI, fig. 12 *a*). In the early stages the mycelium is 3 to 5 μ in diameter and several hundred μ long, branched, and filled with a fine, granular protoplasm. Just before the hyphae break up into segments they enlarge greatly. Most of the segments formed contain two nuclei each. There are, however, some segments cut off that appear to have no nuclei (Pl. XLI, fig. 12 *b*). These empty segments are often found attached to the mature spores. The segments are cut off from the ends of short lateral branches or from the end of the main branch, and the process proceeds backwards until all the mycelium is used. Just before segmentation the tip of the branch enlarges, two nuclei pass in, and are cut off by a cross wall. The segment continues to enlarge, and begins to round up before it becomes free (Pl. XLI, fig. 12 *b*). At this time the wall thickens and the entire surface becomes gelatinized, so that it is not possible to determine the behavior of the nuclei at this point in the life history. It is very probable that the nuclei fuse at this time, because a little later nearly all the segments are uninucleate. The thick episporium and the

endosporium are now completely formed. In a very few cases, at this stage, spores have been found with two nuclei (Pl. XLI, fig. 12 *e*). In some of the spores the nuclei are very close together, and seem to be in the act of fusing. It is very likely that the mature chlamydospore contains a fusion nucleus (Pl. XLI, fig. 12 *d*).

When the chlamydospore obtained from the host plant germinates, the fusion nucleus passes into the promycelium and divides to form four daughter nuclei (Pl. XL, fig. 9 *a*). One of these nuclei is present in each cell of the promycelium. The sporidia and all the secondary spores formed by budding are uninucleate (Pl. XL, fig. 9 *b*). To sum up, the mycelium in the host is multinucleate and breaks up into binucleate segments: the nuclei apparently fuse before the chlamydospores are mature, the mature chlamydospores thus being uninucleate.

I have previously discussed the fusion of the sporidia of the smuts, and have noted that there may be different degrees of fusion in different species. The work with *Ustilago Heufleri* shows that there is a nuclear and cytoplasmic fusion of the sporidia and of the secondary spores. Each spore resulting from the fusion of sporidia contains two nuclei at first; later, when the spore is mature, one nucleus is present. This same condition exists when the chlamydospores are formed from the hyphae in culture; the segments are at first binucleate, and the nuclei fuse before the spore is mature.

The nuclear condition of the germ tube that enters the host was not determined for *Ustilago Heufleri*. In some smuts, according to Rawitscher (17, p. 687), the mycelium that enters the host is uninucleate, as in *U. Zeae*, and fusion of the short hyphae in the plant gives rise to the binucleate condition. In other smuts, like *U. Hordei*—" *U. carbo*" (17, pp. 691-697), the mycelium that enters the host is binucleate. This latter is probably true in the case of *U. Heufleri*, because at no time has a uninucleate mycelium been found in the tissues of the host, and no fusion of the mycelium at or before the time of spore-formation could be recognized. However, the nucleus in the mycelium may divide without a division of the cell; in this way the cells might become multinucleate without necessarily becoming sporophytic. Lutman (14, p. 1220), says that:

Nuclear division may continue until the cell contains twenty or thirty sister nuclei. The sporophytic generation cannot be assumed to begin until the binucleated condition has been definitely established.

Rawitscher, in all his work, studied the mycelium of the smut when it was uninucleate or distinctly binucleate, as at the time of spore-formation. Lutman (p. 1220), however, in speaking of the nuclear condition of the smuts, says:

In general it may be said at the present time as to the nuclear condition of the smut mycelium that in the genus *Ustilago* the cells are multinucleated up to the time of spore formation; in some of the *Tilletia* group the cells are certainly binucleated while in others they are probably so, at least to the extent that many binucleated cells are present,

The nuclear condition that he found in species of *Ustilago* is the one that exists in the mycelium of *U. Heufleri*. The cells of the mycelium are multinucleate, but with the nuclei in loose pairs. At the time of spore-formation the mycelium breaks up into binucleate segments. These segments become chlamydospores, and the nuclei fuse before the spores are mature. In all the species of smuts studied, the chlamydospores arise from binucleate segments of the mycelium, and there must be an intracellular fusion of the nuclei of the young chlamydospore; for the mature chlamydospore contains a single nucleus.

GENERAL SUMMARY

1. The outstanding result of the writer's work on the smuts is the evidence presented that *Ustilago Hordei*, *U. Triticci*, and *U. Heufleri* can complete their morphological life history saprophytically in artificial culture media. This amplification of the recent work of Kniep with *Urocystis Anemones* leads to the conclusion that careful enough adjustment of the environmental requirements may make it possible to bring about the same results in all the smuts, thus showing that no portion of their life cycle is obligatively parasitic.

2. The development of mycelium, comparable with that of other fungi in culture, depends only, as shown, upon the presence of an appropriate medium and of otherwise optimum conditions. Mycelium is formed by *Ustilago Zeae*, for example, when the secondary spores are placed on glycerin agar; when this mycelium is afterward transferred to a 5-percent maltose solution under special conditions, large amounts of mycelium are formed.

3. The mycelium of *Tilletia Triticci* and of *Tilletia foetans* develops best on a heavy oatmeal agar; malt extract in solution is the best liquid medium, and dextrose forms the best sugar solution for this purpose. The addition of calcium nitrate and potassium nitrate to the solution favors the formation of mycelium, while magnesium sulfate and potassium dihydrogen phosphate inhibit its formation.

4. The formation of sporidia and secondary spores, in the species of *Ustilago* studied, is most abundant on agars containing malt extract, peptone, calcium nitrate, and potassium nitrate; here the two salts inhibit rather than promote mycelial development. Of the sugars, maltose in solution is by far the best in promoting a rapid budding of secondary spores; dextrose, levulose, and lactose have very little effect.

5. Potassium hydrogen phosphate and magnesium sulfate, while acting negatively on *Tilletia*, promote the formation of mycelium and cause the secondary spores of *Ustilago Hordei* and *Ustilago Heufleri* to conjugate and form resting spores, when added to a maltose solution.

6. Resting spores of *Tilletia Triticci* do not form in nutrient solution, but appear when the food supply becomes exhausted. These spores have all the appearance of chlamydospores except that they do not form sporidia on germination, but mycelium and secondary spores only.

7. The smuts studied develop best on a slightly acid medium.
8. Sugars are not fermented by the four species of *Ustilago* studied.
9. The full life history of *Ustilago Heufleri* is here presented for the first time. Its mycelium in the host plant is multinucleate until just preceding spore-formation, when it breaks up into binucleate hyphae that segment and round up into binucleate chlamydospores. The two nuclei in each spore fuse before the chlamydospore is mature. The chlamydospore germinates to produce a 2- -4-septate promycelium, which in turn forms sporidia at the septa.
10. The sporidia and secondary spores of *Ustilago Heufleri* formed in culture are uninucleate. In old cultures they frequently fuse in pairs, the nucleus and cytoplasm of one secondary spore passing into the other; when the food supply becomes exhausted, the mycelium present breaks up into segments which may also fuse in pairs and form chlamydospores. The secondary spores may produce hyphae directly, and these may break up into binucleate segments to form chlamydospores. The chlamydospores from both sources can germinate to form promycelia.
11. The fresh chlamydospores of *Ustilago Heufleri* gathered in spring did not germinate; when, however, they were frozen for a few days and then treated with oxygen for 24 hours, they germinated readily in water, in dextrose solution, and on malt-extract agar.
12. The infection of *Erythronium americanum* by *Ustilago Heufleri* takes place when the leaves of the host are just appearing through the soil. The smut can not infect the leaves that are noticeably above the ground.
13. Infection of wheat by *Tilletia Tritici* occurs from the time of germination until a few days after the germination of the wheat, as reported by others. The germ tube from the fused sporidia enters between the cells of the sheath leaf; it is very seldom that the germ tube is intracellular.
14. The mycelium and secondary spores of *Tilletia Tritici* are not able to infect the host at least at the time and place where infection occurs from the usual infection tube.
15. The mycelium and resting spores of *Ustilago Zeae*, formed in culture, are not able to infect the host.

These investigations were carried on in the cryptogamic laboratory of the University of Michigan under the direction of Prof. C. H. Kauffman, to whom the author wishes to express his gratitude for his interest in the work and his helpful constructive criticisms. I also wish to thank Prof. J. H. Hotson of the University of Washington for materials and helpful criticism.

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For a more complete bibliography of the literature of the smuts the student is referred to the work of Lutman (14).

DESCRIPTION OF PLATES

PLATE XXXIX

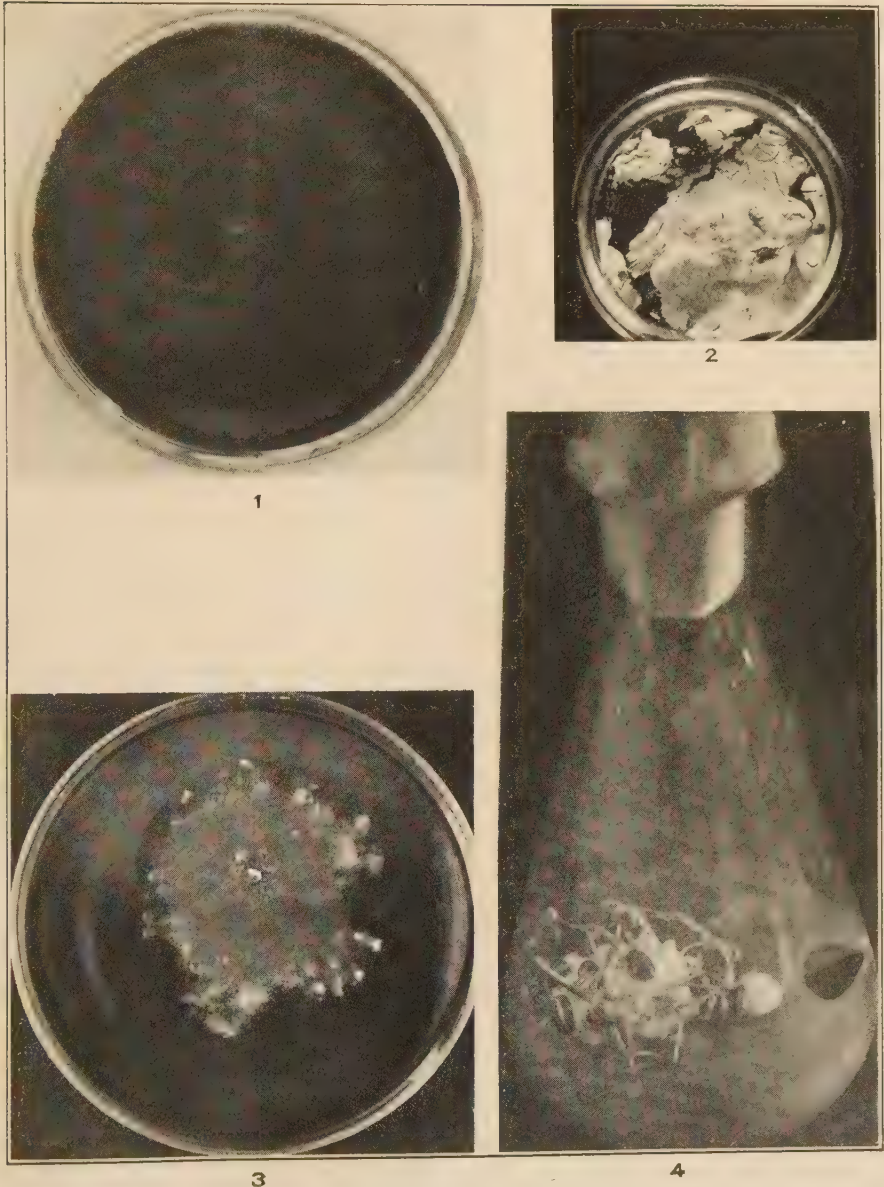
Photographs reduced $\frac{1}{2}$

- FIG. 1. Culture of *Ustilago Heufleri* showing the formation of chlamydospores.
- FIG. 2. The vegetative mycelium of *Tilletia Triticici* developed on sterile wheat kernels.
- FIG. 3. The mycelium of *Ustilago Zeae* formed on glycerin agar.
- FIG. 4. The vegetative mycelium of *Tilletia Triticici* formed on nutrient agar. Sterile wheat seedlings growing on the culture.

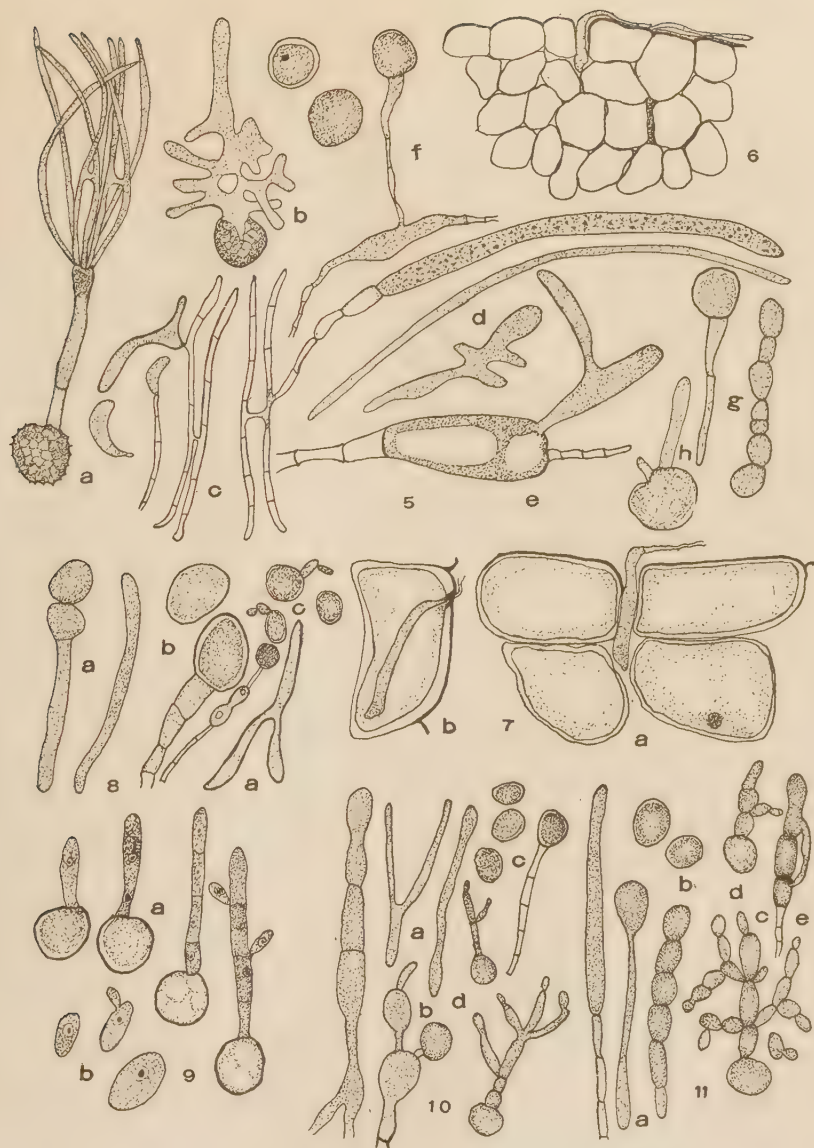
PLATE XL

Bausch and Lomb 15 X ocular and 3-mm. objective were used (X 700), except for figure 6, which was drawn with an 8-mm. objective (X 350).

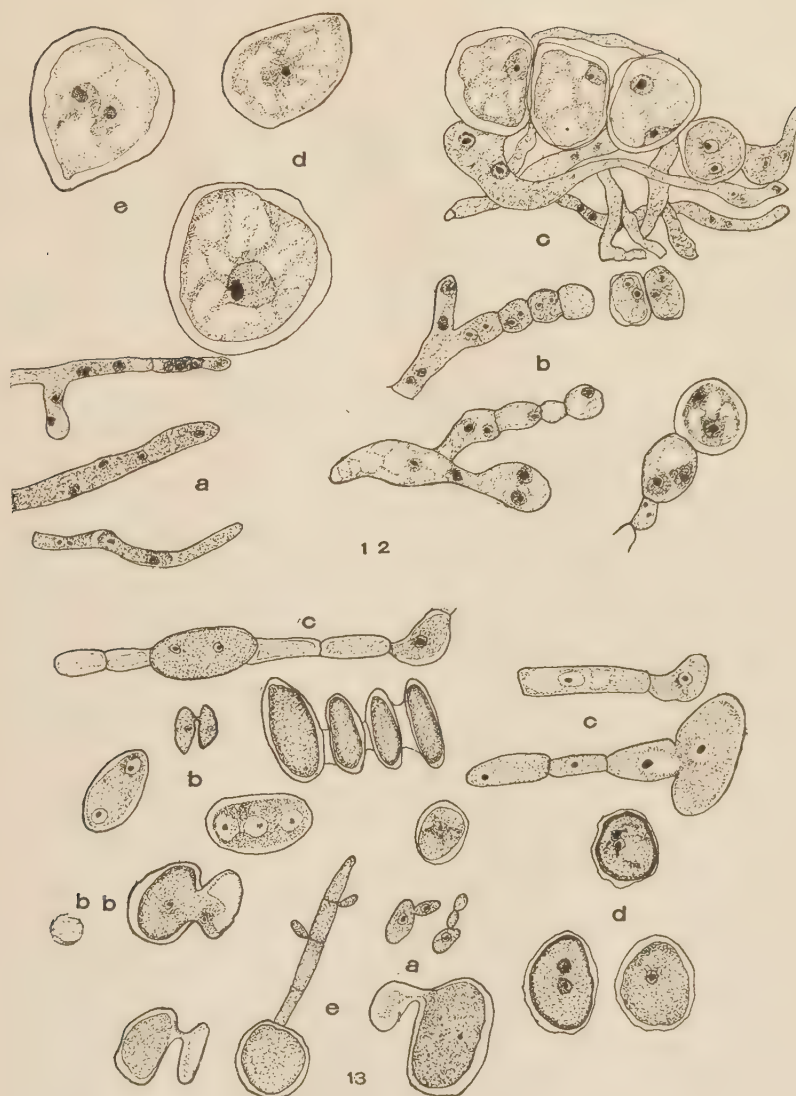
FIG. 5. *a*, Chlamydospore of *Tilletia Triticici* germinated on malt-extract agar. *b*, Abnormal germination of chlamydospores. *c*, Fused sporidia and secondary spores. *d*, Large and small hyphae formed in culture. *e*, A large resting spore germinating. *g*, Oidia obtained from a culture on agar. *f*, *h*, Chlamydospores, formed in culture, germinating.



SARTORIS: LIFE HISTORY AND PHYSIOLOGY OF SMUTS



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FIG. 6. A germ tube of *Tilletia Tritici* entering between the cells of the sheath of a wheat seedling.

FIG. 7. *a*, Same as a portion of figure 6, enlarged. *b*, The germ tube of *Tilletia Tritici* entering a cell of the sheath.

FIG. 8. *a*, Mycelium of *Ustilago Zeae* formed on glycerin agar. *b*, Large resting spores formed at the tip of a hypha. *c*, Small resting spores of the same size as the chlamydospores formed on the host; two are germinating.

FIG. 9. *a*, Stages in the germination of the chlamydospores of *Ustilago Heufleri*. *b*, Uninucleate sporidia and secondary spores.

FIG. 10. *a*, Mycelium of *Ustilago Tritici* formed in culture. *b*, The swollen end of a germ tube. *c*, Chlamydospores formed in culture. *d*, Germination of a chlamydospore formed in culture.

FIG. 11. *a*, Mycelium of *Ustilago Hordei* formed in culture. *b*, Chlamydospores and their formation in culture. *c*, Chlamydospores derived from the host germinating on nutrient agar. *d*, Germination of a chlamydospore formed in culture. *e*, Fusion of two cells in culture.

PLATE XLI

Figure 12 was drawn with a Bausch and Lomb 1.9-mm. oil-immersion objective and a 15 × ocular (× 1,200). Figure 13 was drawn with a 3-mm. objective and a 15 × ocular (× 700).

FIG. 12. *a*, Multinucleate mycelium of *Ustilago Heufleri* formed in a leaf of *Erythronium americanum*. *b*, The mycelium breaking up into binucleate segments. *c*, The segments rounding up to form chlamydospores. *d*, Mature uninucleate chlamydospores. *e*, Germination of a chlamydospore formed in culture.

FIG. 13. *a*, Uninucleate budding secondary spores of *Ustilago Heufleri*. *b* and *bb*, Stages in the conjugation of the secondary spores (from a culture). *c*, Septate mycelium formed on agar media, and the fusion of two of the cells of such a mycelium. *d*, Younger and older chlamydospores (from culture). *e*, Germination of a chlamydospore formed in culture.

